CERTIFICATE OF VERIFICATION

I, Catherine Grosset-Fournier

of

GROSSET-FOURNIER & DEMACHY SARL
54, rue Saint Lazare
F-75009 PARIS
France

hereby declare

1. that I am competent in the French and English languages,

2. that, to the best of my knowledge and belief, the attached document is a true and compete English translation made by me of the PCT/FR2004/050214, and that the said English translation corresponds in all material respects with the French original.

Dated this 2nd day of December 2005

Catherine Grosset-Fournier

Composition comprising the polyprotein NS3/NS4 and the polypeptide NS5b of HCV, expression vectors including the corresponding nucleic sequences and their therapeutic use

The present invention relates to the field of prophylactic and therapeutic vaccination directed against the hepatitis C virus (HCV). It relates in particular to a novel composition containing a polyprotein corresponding to the two colinear proteins NS3 and NS4 (hereafter called polyprotein NS3/NS4) and a polypeptide constituted by NS5b, the vectors, such as adenovirus or poxvirus, capable of expressing this composition and their use as vaccine.

Hepatitis C is the major cause of transfusion-acquired hepatitis. Hepatitis C can also be transmitted by other percutaneous routes, for example by injection of drugs by intravenous route. The risk of contamination of health professionals is moreover not negligible. Sexual transmission has been described.

Hepatitis C differs from other forms of liver diseases associated with viruses, such as hepatitis A, B or D. The infections by the hepatitis C virus (HCV or HCV) are mostly chronic resulting in diseases of the liver, such as hepatitis, cirrhosis and carcinoma in a large number of cases (5 to 20%) and represents 30% of the hepatic transplants in developed countries.

Although the risk of transmission of the virus by transfusion has diminished owing to the introduction of screening tests in the 1990s, the frequency of new HCV infections remains high. By way of example, a recent study indicates that today there are still 10,000 to 15,000 new cases of infection per year in France (S. Deuffic et al., Hepatology 1999; 29: 1596-1601). Currently, approximately 170 million people worldwide are chronically infected by HCV (Hepatitis C: Global prevalence (update), 2000, Weekly Epidemiological Record, Vol 75(3)). The high-risk populations are principally hospital staff and intravenous-drug users, but there are asymptomatic blood donors who do not belong to these high-risk groups and in whom circulating anti-HCV antibodies have been found. For the latter, the infection route has not yet been identified. HCV infections therefore exist (estimated at between 5 and 10%), known as sporadic infections, the etiology of which is unknown and which cannot be controlled.

HCV was the first hepatotropic virus isolated by means of molecular biology techniques. The viral genome sequences were cloned before the viral particle was visualized.

HCV belongs to a new genus of the *Flaviviridae* family, the hepaciviruses. It is a positive single-strand RNA virus, of 9.5 kb, which is replicated by a complementary RNA copy and the translation product of which is a polyprotein precursor of approximately 3,000 amino acids. The 5' end of the HCV genome corresponds to an untranslated region adjacent to the genes that code for the structural proteins, the core protein of the nucleocapsid, the two envelope glycoproteins, El and E2, and a small protein called p7. The 5' untranslated region and the gene core are relatively well preserved in the different genotypes. The envelope proteins El and E2 are encoded by regions that are more variable from one isolate to another. The protein p7 is an extremely hydrophobic protein, which may constitute an ion channel. The 3' end of the HCV genome contains the genes that code for the non-structural proteins (NS2, NS3, NS4, NS5) and for a 3' non-coding region possessing a well-conserved domain (Major ME, Feinstone SM, Hepatology, June 1997,25 (6): 1527-1538).

At present, the most effective therapy for the treatment of hepatitis C combines pegylated interferon and ribavin (Manns MP et al., The Lancet, 22nd September 2001, Vol. 358, 958-965). Whilst this therapy is particularly effective in the case of patients infected by viral strains belonging to the genotypes 2 and 3, it still has only a limited effect on the genotypes 1a, 1b and 4 (Manns MP, *op. cit.*). Less than 50% of the treated patients become "long-term responders". Moreover, this therapy is an expensive intervention (10,000 to 15,000 euros/patient/year) and is associated with toxic effects. In fact, 5 to 10% of the patients are obliged to stop treatment before the end.

It is therefore necessary to develop a vaccine composition targeting all the genotypes.

Several studies now show that the control of an infection caused by HCV either naturally (spontaneous resolution), or after treatment (therapeutic resolution) is associated with the induction or potentialization of cell-mediated immune responses involving the T-CD4$^+$ and T-CD8$^+$ lymphocytes (as described for example in LECHNER, F. et al., Eur. J. Immunol., 30: 2479-

2487 (2000) and in Thimme R. et al., 2001, J. Exp. Med., 194 (10): 1395-1406).

The molecules of the major histocompatibility complex (MHC, also known as HLA in humans) are referred to as class I or class II. The class I molecules are expressed on virtually all of the nucleated cells and are able to present epitopes or peptides to the $CD8^+$ cytotoxic T lymphocytes (CTL). The class II molecules are able to present epitopes to the $CD4^+$ T cells, but their expression is restricted to antigen-presenting cells.

The vaccines against the hepatitis C virus currently envisaged are based on the use of adjuvant recombinant proteins, peptides, expression vectors among which there can be mentioned vectors of viral or bacterial origin or of naked DNA. In this case, one or more viral proteins or one or more genes coding for these viral proteins are used.

When several viral proteins or one or more genes coding for these viral proteins are selected, the latter are often constituted either by some or all of the structural proteins (Makimura et al., 1996, Vaccine, 14: 28-34; Fournilier A. et al., 1999, J. Virology, 73: 7497-7504), or by individual non-structural proteins or comprising at least two contiguous proteins (Brinster et al., 2001, Hepatology, 34: 1206-1217), or by a mixture of structural and non-structural proteins (Pancholi et al., 2003, J. Virology, 77: 382-390).

The Patent Application WO99/38880 describes the use of three genes coding separately for the three proteins NS3, NS4 and NS5 (a and b) in a vaccine composition comprising three DNA vaccines each expressing these three proteins separately. The authors show the induction of T lymphocytes specific to the three antigens in mice. Only the vaccine expressing NS5a and b has been tested *in vivo* in a protection test.

The Patent Application WO01/30812 describes the use of a fusion protein constituted by the non-structural proteins NS3, NS4 and NS5a, if necessary in combination with the non-structural protein NS5b. The authors have indicated that this combination made it possible to activate the HCV-specific T cells. This Patent Application simply describes the ability of vaccine formulations (naked-DNA, recombinant-adenovirus or recombinant-vaccinia-virus type) expressing the fusion protein NS3, NS4, NS5a or the protein NS5a to induce specific immune responses mediated by specific T lymphocytes.

The Applicant has now demonstrated, against all expectation, that the particular combination of the non-structural proteins NS3, NS4 and NS5b, NS3 and NS4 being expressed colinearly had a better immunogenic power and protective power superior to that obtained with a vaccine also including, apart from these non-structural proteins, the protein NS5a and/or other structural proteins of HCV such as core, El or E2, and had an effect on the ability of cells originating from patients infected by viral strains to induce specific immune responses.

Thus, an object of the present invention is a peptide composition comprising a polyprotein NS3/NS4 of the hepatitis C virus, as well as a polypeptide NS5b of the hepatitis C virus.

An object of the invention is also the vectors including the nucleotide sequences coding for this peptide composition, such as the adenoviruses and poxviruses, as well as microorganisms or host cells transformed by these vectors.

An object of the invention is finally the antibodies directed against the peptide composition of the invention, as well as the use of the peptide composition, vectors and antibodies for the preparation of a medicament intended for the inhibition or control of an infection caused by the hepatitis C virus, and in a vaccine composition.

The present invention therefore proposes a novel peptide composition constituted by a polyprotein NS3/NS4 and a polypeptide NS5b of HCV, which composition has the ability to stimulate a cell-mediated immune response specific to HCV, such that it is useful in the field of prophylactic and therapeutic vaccination directed against the hepatitis C virus.

The polyprotein NS3/NS4 of the peptide composition of the invention is constituted by the protein NS3 and the protein NS4a and b, without interruption in the peptide sequence, as in the native polyprotein. In fact, as indicated previously, the HCV genome contains a single open reading frame that is transcribed into a polyprotein. This HCV polyprotein can be cleaved in order to produce at least ten distinct parts, in the order NH_2-Core-El-E2-p7-NS2-*NS3*-*NS4a*-*NS4b*-NS5a-NS5b-COOH.

The protein NS3 is a protein of 630 amino acids, which appears approximately from amino acid 1027 to amino acid 1657 of the polyprotein.

The protein NS4, a protein of 314 amino acids, appears approximately from amino acid 1658 to amino acid 1972 (numbering with respect to HCV-1) (Choo et al., 1991, Proc. Natl. Acad. Sci., vol 88: 2451-2455). The polyprotein NS3/NS4 therefore appears approximately from amino acid 1027 to amino acid 1972.

As regards the polypeptide NS5b also contained in the composition of the invention, it is constituted by 590 amino acids and appears approximately from amino acid 2421 to amino acid 3011 of the polyprotein (Choo et al., 1991, *op. cit.*).

The protein NS3 comprises two distinct structural domains, namely an N-terminal domain endowed with an active serine protease activity that is involved in the maturation of the viral polyprotein, and a C-terminal domain comprising a helicase activity associated with an NTPase activity that plays a role in the replication of the viral genome.

By "polyprotein NS3/NS4" and "polypeptide NS5b", is of course meant the polyproteins and polypeptides having the native amino acid sequences, originating from any HCV strain and isolate, as well as their analogues, muteins and homologues.

By "analogues" or "muteins" of the polyprotein and of the polypeptide, is meant the biologically active derivatives of the reference molecules that have the desired activity, namely the ability to stimulate a cell-mediated immune response as defined above.

Generally, the term "analogue" refers to compounds having a native polypeptide sequence and structure having one or more additions, substitutions (generally conservative in terms of nature) and/or amino acid deletions, relative to the native molecule, to the extent that the modifications do not destroy the immunogenic activity. By the term "mutein", is meant the peptides having one or more elements imitating the peptide (peptoids), such as those described in the Patent Application PCT WO91/04282. Preferably, the analogue or the mutein have at least the same immunoactivity as the native molecule. Processes for preparing polypeptide analogues and muteins are known to a person skilled in the art and are described below.

The particularly preferred analogues include substitutions that are conservative in nature, i.e. the substitutions, which take place in a family of

amino acids. Specifically, the amino acids are generally divided into 4 families, namely (1) the acid amino acids such as aspartate and glutamate, (2) the basic amino acids such as lysine, arginine and histidine, (3) the non-polar amino acids such as alanine, leucine, isoleucine, proline, phenylalanine, methionine and tryptophane and (4) the polar non-charged amino acids such as glycine, asparagine, glutamine, cysteine, serine, threonine and tyrosine. Phenylalanine, tryptophane and tyrosine are sometimes classified as aromatic amino acids. For example, it can reasonably be predicted that an isolated replacement of leucine by isoleucine or valine, of an aspartate by a glutamate, of a threonine by a serine, or a similar conservative replacement of one amino acid by another amino acid having a structural relationship, will not have a major effect on the biological activity. A person skilled in the art will easily determine the regions of the peptide molecule of interest that can tolerate a change by referring to the Hopp/Woods and Kyte-Doolittle plots, well known in the art.

By "homology", is meant the percentage of identity between two peptide molecules, such as polyproteins and polypeptides. Two amino acid sequences are "more or less homologous" to each other when the sequences have at least 60%, preferably at least 75%, more preferably also at least 80-85%, more preferably also at least 90% and still more preferably at least 95-98% or more of sequence identity over a defined length of the peptide molecules.

Generally, the term "identity" refers to an exact amino acid to amino acid correspondence of two peptide sequences. The percentage of identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of mismatches between the two aligned sequences, dividing by the length of the shorter sequence and multiplying the result by 100. The percentage of identity can also be determined using computer programs such as ALIGN, Dayhoff, M. O. in Atlas of Protein Sequence and Structure M. O. Dayhoffed., 1981, 5 Suppl., 3: 482-489.

The nucleic acid and amino acid sequences of a certain number of HCV strains and isolates, and in particular of the protein NS3, of the protein NS4 and of the polypeptide NS5b, have already been determined.

For example, the isolate HCV-J1 is described in Okamoto H. et al.,1992, Nucleic Acids Res., 20: 6410-6410. The complete coding sequences of two independent HCV isolates, namely the isolates HCV-J and-BK, have been described in Kato et al., 1990, Proc. Natl. Acad., Sci., 87: 9524-9528 and in Takamizawa et al., 1991, J. Virol., 65: 1105-1113 respectively. As regards the isolate HCV-1, it is described in Choo et al., 1990, Brit. Med. Bull., 46: 423-441 and in Choo et al., 1991, *op. cit.*. The isolate HVC-H has been described in Inchauspe G. et al;, 1991, Proc. Natl. Acad. Sci., 88: 10292-10296. The isolate HCV-G9 has been described in Okamoto H., et al., 1994, J. Gen. Virol., 45: 629-635. The isolates HCV-J6 and -J8 have been described in Okamoto H., et al., 1991, J. Gen. Virol., 72: 2697-2704 and Okamoto H., et al., 1992, Virology, 188: 331-341 respectively. The isolate HVC-BEBE1 has been described in Nako H., et al., 1996, J. Gen. Virol., 141: 701-704 and the isolate HCV-NZL1 has been described in Sakamoto M., et al., 1994, J. Gen. Virol., 75: 1761- 1768. As regards the isolate HCV-Tr, it has been described in Chayama K., et al., 1994, J. Gen. Virol., 75: 3623-3628. The isolates HCV-ED43 and -EUH1480 have been described in Chamberlain R. W., et al., 1997, J. Gen. Virol., 78: 1341-1347 and Chamberlain R. W., et al., 1997, Biochem. Biophys. Res. Commun., 236: 44-49 respectively. The isolate HCV-EUHK2 has been described in Adams A., et al., 1997, Biochem. Biophys. Res. Commun., 234: 393-396. The isolates HCV-VN235, -VN405 and -VN004 have been described in Tokita H., et al., 1998, J. Gen. Virol., 79: 1847. Finally, as regards the isolates HCV-JK049 and-JK046, they have been described in Tokita H. et al., 1996, J. Gen. Virol., 77: 293-301.

The HCV strains and isolates, as illustrated above, can have different genotypes, namely genotypes 1a (isolates HCV-1,-J1 and-H), 1b (isolates HCV- J and BK), 1c (isolate HCV-G9), 2a (isolate HCV-J6), 2b (isolate HCV-J8), 2c (isolate HCV-BEBE1), 3a (isolate HCV-NZL1), 3b (isolate HCV-Tr), 4a (isolate HCV-ED43), 5a (isolate HCV-EUH1480), 6a (isolate HCV-EUHK2), 7b (isolate HCV-VN235), 8b (isolate HCV-VN405), 9a (isolate HCV-VN004), 10a (isolate HCV-JK049) and 11a (isolate HCV-JK046).

According to one embodiment of the invention, NS3 and/or NS4 and/or NS5b originate from viruses of different genotypes.

According to another embodiment, NS3 and/or NS4 and/or NS5b originate from viruses of the same genotype, preferably of genotype 1b.

The polyprotein NS3/NS4 and the polypeptide NS5b contained in the peptide composition of the invention can be either of native origin, or of recombinant origin.

The polyprotein NS3/NS4 and the polypeptide NS5b of native origin are obtained from HCV strains or isolates, by means of the use of synthetic oligonucleotide primers that will serve to amplify the native viral sequences, either from sera of patients infected by the targeted viral genotype or genotypes, or from already purified viral RNA, originating for example from patients' blood or liver, or from complementary DNA that is free or cloned beforehand in an expression vector, or also from viral particles purified from biological samples or *in vitro* propagation system.

The polyprotein NS3/NS4 and the polypeptide NS5b of the invention of recombinant origin can also be obtained by the genetic engineering technique, which comprises the steps of:

- culture of a microorganism or of eukaryotic cell(s) transformed using a nucleotide sequence coding for said polyprotein NS3/NS4 or for said polypeptide NS5b and
- recovery of the peptide produced by said microorganism or said eukaryotic cells.

This technique is well known to a person skilled in the art. For more details concerning this, reference can be made to the following work: Recombinant DNA Technology I, Editors Ales Prokop, Raskesh K Bajpai; Annals of the New-York Academy of Sciences, Volume 646, 1991.

The nucleotide sequences coding for the polyprotein NS3/NS4 and the polypeptide NS5b can be prepared by chemical synthesis in conjunction with a genetic engineering approach or by genetic engineering alone, using the techniques well known to a person skilled in the art and described for example in Sambrook J. et al., Molecular Cloning: A Laboratory Manual, 1989.

The nucleotide sequences coding for the polyprotein NS3/NS4 and the polypeptide NS5b can be inserted into expression vectors in a suitable expression system, in order to obtain the peptide composition of the invention.

Of course, the nucleotide sequences can be inserted into a single expression vector or into two different expression vectors. In the latter case, the sequence coding for the polyprotein NS3/NS4 is inserted into one of the two vectors and the sequence coding for the polypeptide NS5b is inserted into the other vector, these two vectors being either identical or different in nature.

Thus, another object of the invention is the expression vectors comprising a nucleotide sequence coding for the polyprotein NS3/NS4 and a nucleotide sequence coding for the polypeptide NS5b, as well as the means necessary to its expression.

By means necessary to the expression of a peptide is meant, the term peptide being used for any peptide molecule, such as protein, polyprotein, polypeptide, etc., any means that make it possible to obtain the peptide, such as in particular a promoter, a transcription terminator, a replication origin and preferably a selection marker.

The means necessary to the expression of a peptide are operationally linked to the nucleic acid sequence coding for the peptide of interest. By "operationally linked", is meant a juxtaposition of said elements necessary to the expression and of the gene coding for the peptide of interest, which are in a relationship such that it is possible for them to function in an expected manner. For example, additional bases can exist between the promoter and the gene of interest to the extent that their functional relationship is preserved.

The means necessary to the expression of a peptide can be homologous means, i.e. included in the genome of the vector used, or be heterologous. In the latter case, said means are cloned with the peptide of interest to be expressed.

Examples of heterologous promoters include (i) the viral promoters such as the SV40 promoter (simian virus 40), the promoter of the thymidine-kinase gene of the herpes simplex virus (TK-HSV-1), the LTR of the Rous sarcoma virus (RSV), the immediate first promoter of the cytomegalovirus (CMV) and the adenovirus major last promoter (MLP), as well as (ii) any cell promoter that controls the transcription of the genes coding for peptides in upper eukaryotes, such as the constitutive promoter of the diphosphoglycerate-kinase gene (PGK) (Adra et al., 1987, Gene, 60: 65-74), the promoter of the liver-specific alpha-1 antitrypsin and FIX genes and the SM22 promoter

specific to the smooth muscle cells (Moessler et al., 1996, Development, 122: 2415-2425).

According to one embodiment of the invention, the nucleotide sequences coding for said polyprotein NS3/NS4 and said polypeptide NS5b originate from different genotypes.

According to another embodiment, the nucleotide sequences coding for said polyprotein and said polypeptide originate from a virus of the same genotype, preferably genotype 1b.

Here too, by "nucleotide sequence" is meant all the sequences coding for the native polyprotein NS3/NS4 and the native polypeptide NS5b, as well as for their analogues, muteins and homologues, as defined previously.

Said sequences contained in the expression vector can be directly interlinked under the control of a single promoter and/or of a single expression-regulating element, or they can be separate, each being dependent on expression promoters and/or regulators that are independent identical or different.

As expression vectors that are suitable for the purposes of the invention, there can be mentioned for example plasmids, adenovirus-type viral vectors, poxviruses, vaccinia viruses, baculoviruses, salmonella-type bacterial vectors, BCG.

Adenoviruses have been detected in numerous animal species, do not integrate and are only slightly pathogenic. They are capable of infecting a variety of cell types, cells in division and cells at rest. They possess a natural tropism for the bronchial epithelia. Moreover, they have been used as live enteric vaccines for many years with an excellent safety profile. Finally, they can easily be made to grow and be purified in large amounts. These characteristics have meant that the adenoviruses are particularly appropriate for use as expression vectors and in particular as gene therapy vectors for therapeutic purposes and for vaccines.

According to a preferred embodiment, the vector of the invention is an adenovirus.

Examples of adenoviruses to be used in the present invention can be derived from any source of human or animal origin, in particular of canine origin (for example CAV-1 or CAV-2; reference Genbank CAV1GENOM and

CAV77082 respectively), of avian origin (reference Genbank AAVEDSDNA), of bovine origin (such as BAV3, Seshidhar Reddy et al., 1998, J. Virol., 72: 1394-1402), of ovine, feline, porcine origin, of simian origin, or from one of their hybrids. Any serotype can be used. However, adenoviruses of human origin are preferred and in particular adenovirus 5 (AdIV).

Generally, the mentioned viruses are available from the ATCC collections and have been the subject of numerous publications describing their sequence, their organization and their biology, which allows a person skilled in the art to use them easily. For example, the sequence of the adenovirus type 5 is described in the Genbank database (M73260 and M29978) and is incorporated here by way of reference.

The genome of the adenovirus is constituted by a double-strand linear DNA molecule of approximately 36 kb carrying more than approximately 30 genes necessary for terminating the viral cycle. The first genes are divided into 4 regions dispersed in the genome of the adenovirus (E1 to E4). The E1, E2 and E4 regions are essential for viral replication. The E3 region is considered as a non-essential region on the basis of the observation that mutant viruses appear naturally or the hybrid viruses having lost this E3 region continue to replicate like wild-type viruses in cultured cells (Kelly and Lewis, 1973, J. Virol., 12: 643-652). The last genes (L1 to L5) mostly code for the structural proteins constituting the viral capsid. They overlap at least in part the first transcription units and are transcribed from a single promoter (MLP for Major Late Promoter). Moreover, the adenoviral genome carries at the two ends of the cis-acting regions essential for DNA replication, the 5' and 3' inverted terminal repeats (ITRs) and a packing sequence respectively.

The adenoviruses currently used in gene therapy protocols are stripped of the majority of the E1 region, which renders the viruses deficient at the level of their replication in order to avoid their dissemination in the environment and in the host organism. Moreover, most of the adenoviruses are also stripped of the E3 region in order to increase their cloning capacity. The feasibility of gene transfer using these vectors has been demonstrated in a variety of tissues *in vivo* (see for example Yei et al., 1994, Hum. Gene Ther.,5: 731-744; Dai et al., 1995, Proc. Natl. Acad Sci. USA, 92: 1401-1405; US6,099,831; and US6,013,638).

Preferably, the promoters used in the adenoviruses as expression vectors are heterologous promoters such as the CMV and SV40 promoters.

Preferably also, the CMV promoter is the promoter of the polyprotein NS3/NS4 and the expression vector comprises as nucleotide sequence coding for said polyprotein the expression cassette CMV-NS3-NS4.

By "expression cassette", is meant a DNA sequence containing a promoter and an open reading frame for the expression of the peptide of interest, to be inserted into a vector.

Preferably also, the SV40 promoter is the promoter of the polypeptide NS5b and the expression vector comprises as nucleotide sequence coding for said polypeptide the expression cassette SV40- NS5b.

According to one embodiment of the invention, the genome of the adenovirus is modified so as to replace the EI region by the expression cassette CMV-NS3-NS4 and to replace the E3 region by the expression cassette SV40- NS5b.

The methods of suppression and of insertion of DNA sequences into expression vectors are widely known to a person skilled in the art and consist in particular of steps of enzymatic digestion and ligation.

Another expression vector particularly appropriate for the purposes of the invention is a poxvirus, which constitutes another embodiment of the invention.

The poxviruses constitute a group of enveloped complex viruses, differing principally in their unusual morphology, their large DNA genome and their cytoplasmic replication site. The genome of several elements of the *poxviridae*, comprising the Copenhagen strain of the vaccinia virus (VV) (Goebel et al., 1990, Virol. 179: 247- 266 and 517-563) and the modified vaccinia virus Ankara (MVA) strain (Antoine et al., 1998, Virol., 244: 635-396), has been mapped and sequenced. The VV strain possesses a double-strand DNA genome of approximately 192 kb coding for approximately 200 proteins approximately 100 of which are involved in the assembly of the virus. The MVA strain is a highly attenuated strain of vaccinia virus, generated by more than 500 passages in series of the vaccinia virus Ankara strain (CVA) over chicken embryo fibroblasts (Mayr et al., 1975, Infection, 3: 6-16). The MVA virus has been deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) under Number I-721. The determination of the

complete sequence of the MVA genome and comparison with that of the VV allows precise identification of the alterations that have appeared in the viral genome and the definition of seven deletions (I to VII) and of numerous mutations leading to fragmented open reading frames (Antoine et al., 1998, Virology, 244: 365-396).

Other examples of poxviruses that are appropriate for the purposes of the invention include duck pox, fowl pox, cow pox, entomopox, monkey pox, swine pox and penguin pox.

The poxvirus is found in two morphologically distinct forms, called intracellular mature virus (IMV) and enveloped extracellular virus (EEV).

The poxvirus used as an expression vector of the invention has at least one of the following characteristics, taken alone or in combination:

(i) the poxvirus is an MVA virus,

(ii) the poxvirus is in the IMV morphological form, and

(iii) the genome of the poxvirus is modified so as to insert the expression cassette NS3/NS4 and to insert the expression cassette NS5b.

When the genome of the poxvirus is modified so as to insert the two cassettes of interest, the means necessary to their expression are homologues. Thus, in the case where the MVA virus is used, the expression of NS3/NS4 can be for example under the control of the promoter ph5r so that the corresponding expression cassette is ph5r-NS3-NS4, and the expression of NS5b can be for example under the control of the promoter p7.5 so that the corresponding expression cassette is p7.5- NS5b, and vice versa.

According to a particular embodiment, when the genome of the poxvirus is modified so as to insert the two cassettes of interest, the two said expression cassettes are oriented in the same direction.

According to another particular embodiment, they are oriented in the opposite direction.

Here too, the expression cassettes are inserted into the genome of the poxvirus in a manner known to a person skilled in the art, as indicated previously.

The vectors of the invention can also comprise sequences necessary for targeting peptides towards particular cell compartments. An example of targeting can be the targeting towards the endoplasmic reticulum obtained

using address sequences of the leader sequence type originating from the protein E3 of the adenovirus (Ciernik I. F., et al., The Journal of Immunology, 1999, 162, 3915-3925).

They can also comprise sequences necessary for targeting towards the dendritic cells and for targeting at the membrane of the cells.

An object of the invention is also the microorganisms and the eukaryotic cells transformed by an expression vector of the invention.

By way of examples of microorganism that are suitable for the purposes of the invention, there can be mentioned the yeasts, such as those of the following families: *Saccharomyces, Schizosaccharomyces, Kluveromyces, Pichia, Hanseluna, Yarrowia, Schwantomyces, Zygosaccharomyces, Saccharomyces cerevisiae, Saccharomyces carlsbergensis* and *Kluveromyces lactis* being preferred; and the bacteria, such as E. coli and those of the following families: *Lactobacillus, Lactococcus, Salmonella, Streptococcus, Bacillus* and *Streptomyces*.

By way of examples of eukaryotic cells, there can be mentioned cells originating from animals such as mammals, reptiles, insects and equivalent. The preferred eukaryotic cells are cells originating from the Chinese hamster (CHO cells), monkey (COS and Vero cells), baby hamster kidney (BHK cells), pig kidney (PK 15 cells) and rabbit kidney (RK13 cells), human osteosarcoma cell lines (143 B), HeLa human cell lines and the human hepatoma cell lines (Hep G2-type cells), as well as insect cell lines (for example of Spodoptera frugiperda).

The host cells can be provided in cultures in suspension or in flasks, in tissue cultures, organ cultures and equivalent. The host cells can also be transgenic animals.

The invention also relates to antibodies directed against one of the peptide compositions of the invention as defined previously or against one of the expression vectors of the invention as defined previously.

The antibodies according to the invention are either polyclonal or monoclonal antibodies.

The abovementioned polyclonal antibodies can be obtained by immunization of an animal with the peptide composition of the invention or with the vector of the invention as "antigen of interest", followed by the

recovery of the antibodies sought in purified form, by sampling the serum of said animal, and separation of said antibodies from the other constituents of the serum, in particular by affinity chromatography on a column to which is fixed an antigen specifically recognized by the antibodies, in particular a viral antigen of interest.

The monoclonal antibodies can be obtained by the hybridomas technique the general principle of which is recalled hereafter.

In a first step, an animal, generally a mouse, (or cells in culture within the framework of *in vitro* immunizations) is immunized with the peptide composition of the invention or with the vector of the invention as "antigen of interest", the B lymphocytes of which are then capable of producing antibodies against said antigen. These antibody-producing lymphocytes are then fused with "immortal" myelomatous cells (murine in the example) in order to produce hybridomas. From the thus-obtained heterogeneous mixture of cells, a selection is then made of cells capable of producing a particular antibody and multiplying indefinitely. Each hybridoma is multiplied in clone form, each leading to the production of a monoclonal antibody the recognition properties of which vis-à-vis the antigen of interest can be tested for example by ELISA, by immunotransfer in one or two dimensions, by immunofluorescence, or using a biocaptor. The monoclonal antibodies thus selected are subsequently purified in particular according to the affinity chromatography technique described above.

The peptide compositions, the expression vectors, the nucleotide sequences coding for said polyprotein NS3/NS4 and said polypeptide NS5b, as well as the antibodies of the invention are particularly effective for the inhibition, prevention and control of the infection of patients carrying the HCV virus, so that their use for the preparation of a medicament constitutes another object of the invention.

The present invention also relates to a pharmaceutical composition, in particular a vaccine, containing as active ingredient the peptide composition of the invention, or an expression vector of the invention, or an expression vector comprising a nucleotide sequence coding for the polyprotein NS3/NS4 with an expression vector comprising a nucleotide sequence coding for the polypeptide NS5b, or the nucleotide sequences coding for said polyprotein

NS3/NS4 and said polypeptide NS5b, said nucleotide sequences corresponding to the sequences contained in the expression vectors of the invention, placed under the control of elements necessary to an expression constitutive of and/or inducible from said peptides, or at least one of the antibodies of the invention.

By elements necessary to an expression constitutive of the peptides, is meant a promoter that is ubiquitous or specific to the eukaryotic cells.

As elements necessary to an expression inducible from the peptides, there can be mentioned the elements of regulation of the operon of *E. coli* for tetracycline resistance (Gossen M. et al., Proc Natl Acad Sci USA, 89: 5547-5551 (1992).

According to a particular embodiment of the invention, the pharmaceutical composition also contains a pharmaceutically appropriate vehicle. Of course, a person skilled in the art will easily determine the nature of the pharmaceutically appropriate vehicle and the quantity of polypeptides to be used as a function of the constituents of the pharmaceutical composition.

The quantity and nature of the pharmaceutically appropriate vehicle can be easily determined by a person skilled in the art. They are chosen according to the desired pharmaceutical form and method of administration.

The pharmaceutical compositions of the invention are appropriate for oral, sublingual, sub-cutaneous, intramuscular, intravenous, topical, local, intratracheal, intranasal, transdermal, rectal, intraocular, intra-auricular administration, said active ingredient being able to be administrated in a unitary dosage form of administration.

The unitary dosage forms of administration can be for example tablets, gelatin capsules, granules, powders, solutions or injectable oral suspensions, transdermal patches, forms of sublingual, buccal, intratracheal, intraocular, intranasal, intra-auricular or by inhalation administration, forms of topical, transdermal, sub-cutaneous, intramuscular or intravenous administration, forms of rectal administration, or implants. For topical administration, creams, gels, ointments, lotions or collyriums can be envisaged.

These galenic forms are prepared according to the usual methods of the fields considered.

Said unitary dosage forms are dosed in order to allow daily administration of 0.001 to 10 mg of active ingredient per kg of body weight, according to the galenic form.

There may be particular cases where higher or weaker dosages are appropriate; the scope of the invention is not exceeded by such dosages. According to usual practice, the dosage appropriate to each patient is determined by the doctor according to the method of administration, the weight and the response of the patient.

According to another embodiment of the invention, the present invention also relates to a method of treatment of the pathologies associated with the hepatitis C virus, which comprises the administration, to a patient, of an effective dose of a medicament of the invention.

The pharmaceutical compositions of the invention preferably contain as active ingredient one of the vectors of the invention or an expression vector comprising a nucleotide sequence coding for the polyprotein NS3/NS4 with an expression vector comprising a nucleotide sequence coding for the polypeptide NS5b, so that they are useful in prophylactic and therapeutic vaccination.

Prophylactic and therapeutic vaccination can be implemented by injection of a vaccine based on one or more expression vectors of the invention, to the extent that the expression vector or vectors finally code for the polyprotein NS3/NS4 and for the polypeptide NS5b as active ingredient, said injection being or being not followed by boosters. It can also be implemented by injecting two different types of expression vectors of the invention, firstly an adenovirus, then a poxvirus, simultaneously or at different times, and vice versa.

These vectors can be contained in a pharmaceutical kit.

Also, another object of the invention is pharmaceutical kits, in particular vaccinal, comprising at least one expression vector comprising a nucleotide sequence coding for the polyprotein NS3/NS4 and at least one expression vector comprising a nucleotide sequence coding for the polypeptide NS5b.

Another object of the invention is pharmaceutical kits, in particular vaccinal, comprising at least one expression vector of adenovirus type as

defined previously and/or at least one expression vector of poxvirus type as defined previously.

Prophylactic and therapeutic vaccination can also be implemented by injection of a vaccine based on at least one expression vector of the invention, or an expression vector comprising a nucleotide sequence coding for the polyprotein NS3/NS4 with an expression vector comprising a nucleotide sequence coding for the polypeptide NS5b, and at least one pharmaceutical composition of the invention constituted by the peptide composition of the invention or the antibodies of the invention. It can also be implemented by injection of a vaccine based on at least one expression vector of the invention, or an expression vector comprising a nucleotide sequence coding for the polyprotein NS3/NS4 with an expression vector comprising a nucleotide sequence coding for the polypeptide NS5b, and at least one nucleotide sequence coding for the polyprotein NS3/NS4 and for the polypeptide NS5b.

Also, another object of the invention is pharmaceutical kits, in particular vaccinal, comprising at least one expression vector of the invention, or an expression vector comprising a nucleotide sequence coding for the polyprotein NS3/NS4 with an expression vector comprising a nucleotide sequence coding for the polypeptide NS5b, and at least one pharmaceutical composition of the invention or at least one nucleotide sequence coding for the polyprotein NS3/NS4 and for the polypeptide NS5b.

The present invention will be better understood using the following examples that are given only by way of illustration, and are non-limitative, as well as using the attached Figures 1 to 7, in which:

- Figure 1A to 1K represents the maps of the different plasmids used for obtaining an adenovirus AdNS3NS4NS5b according to the invention, on which are indicated the sites of the different restriction enzymes and the location of the sequence fragments coding for NS3/NS4 and for NS5b,
- Figure 2A to 2H represents the maps of the different plasmids used for obtaining a poxvirus MAV NS3NS4NS5b according to the invention, on which are indicated the sites of the different restriction enzymes and the location of the sequence fragments coding for NS3/NS4 and pour NS5b,

- Figure 3 gives the cell response induced by the adenovirus AdNS3NS4, either according to the CTL test (Figure 3A) where the epitope GLL was used for stimulating the splenocytes in culture and for loading the CTL targets and the result of which is expressed as a specific lysis percentage as a function of the effector/target ratio, or according to the ELISPOT test (Figure 3B), specific to the epitope GLL, where the result is given in numbers of spots/10^6 cells,
- Figure 4 gives the cell response induced by the adenovirus AdNS5b according to the test ELISPOT, specific to the epitopes ALY and KLP,
- Figure 5 gives the cell response induced by the adenovirus AdCEIE2 according to the CTL test where the epitope DLM was used for stimulating the splenocytes in culture and for loading the targets of the CTL and the result of which is expressed as a specific lysis percentage as a function of the effector/target ratio,
- Figure 6 gives the titre of the recombinant vaccinia virus, resulting from the trial test, in pfu/ml/mg ovary, for the 4 groups of 8 mice immunized by the different combinations of adenovirus: AdNS3NS4 +AdNS5b (lst group), the adenoviruses AdNS3NS4 + AdNS5b +AdNS5a (2nd group), the adenoviruses AdNS3NS4 + AdNS5b+AdCEIE2 (3rd group) and the adenovirus AdβGal (4th group) and
- Figure 7 gives the titre of the recombinant vaccinia virus, resulting from the trial test, in pfu/ml/mg ovary, for the 3 groups of 8 mice immunized by the following different combinations of adenovirus: AdNS3NS4NS5b (1st group), AdNS3NS4 + AdNS5b (2nd group) and AdβGal (3rd group).

Example 1: Preparation of an adenovirus allowing the expression of the proteins NS3/NS4 and NS5b according to the invention

1. Adenovirus

The recombinant adenoviruses are generated by transfection (CaPO3) of the complementation line 293 (Graham, Smiley, et al. 1977) after linearization of the genomes by *Pac*I. The recombinant viruses propagate and are amplified on this same line, and their purification is carried out from the infected cells. The cells are recovered by centrifugation (1500 rpm, 10

minutes) and lysed by 3 freeze/thaw cycles. The cell lysate is clarified by two centrifugations (2000 rpm, 10 minutes; 8000 rpm, 15 minutes), then purified by two successive ultracentrifugations. The first is carried out on a Caesium Chloride gradient (densities 1.4 and 1.25) at 30,000 rpm for 1 hour. The second is carried out on a Caesium Chloride cushion (density 1.34) at 35,000 rpm for 18 hours. The phases containing the virions are removed and diluted by half in a 60% saccharose buffer. The viral suspensions are then dialysed against formulation buffer (for 10 litres: 3423 g of saccharose; 12.11g of Tris; 2.033 g of $MgCl_2$; 87.7g of NaCl), then aliquoted. Their titration is carried out by indirect immunofluorescence on 293 cells infected by different viral dilutions and marked by an antibody specific to the adenoviral DNA-Binding Protein (α72K B6-8) (Reich, Sarnow, et al. 1983).

2. Preparation of the adenovirus AdNS3NS4

This adenovirus allows the expression of the gene coding for the polyprotein NS3/NS4 (SEQ ID No. 1 and 2) under the control of the CMV promoter.

2.1 PCR Amplification of the nucleotide sequence coding for the polyprotein NS3/NS4

In order to do this, the following oligonucleotides were used:

olV166: 5'-GGG GGG GCT ATG GCG CCT ATC ACG GCC TA-3' (SEQ ID No. 9)

olV171: 5'-GGG GGG ACG CGT TTA GCA TGG CGT GGA GCA GT-3' (SEQ ID No. 10)

as well as the following reagents:

Taq DNA Polymerase, PCR buffer, MgCl,1.5mM and dNTP 10mM (Invitrogen).

The PCR conditions were the following:

5 minutes at 94°C, then

30 cycles of the series: 45 seconds at 94°C, 45 seconds at 62°C and 1 minute at 72°C, then

10 minutes at 72°C

2.2 Insertion of the PCR fragment NS3/NS4 into the transfer plasmid pTG13387

The following stages were carried out:

- Enzymatic digestion of the plasmid pTG13387 (Figure 1A, Transgene) by *Nhe*I/*Mlu*I (*Nhe*I, Invitrogen in React 4 Buffer and *Mlu*I, Invitrogen in React 3 Buffer)
- Enzymatic digestion of the fragment NS3/NS4 by *Nhe*I/*Mlu*I
- Ligation (T4 DNA Ligase (Invitrogen) in Reaction Buffer (Invitrogen)),
- Bacterial transformation (strain 5K, (Transgene)
- Selection of bacterial clones on LB medium (Difco) + ampicillin (100 µg/ml, Duchefa)
- Plasmid maxi-preparation (Qiagen, according to manufacturer's protocol) of a positive clone after restriction analysis
- Restriction analysis: digestion by *Sma*I (Invitrogen in React 4 Buffer) and obtaining of fragments of: 5450, 2164, 909, 214 and 180 pb
- Obtaining of the plasmid pIV315 deleted from its E1 region and containing the sequence NS3/NS4 under the control of the CMV promoter (Figure 1B).

2.3 Homologous recombination with the complete adenoviral genome deleted from its E3 region contained in the plasmid pTG6624

The following stages were carried out:

- Enzymatic digestion of the plasmid pIV315 obtained above by *Pac*I/*Pvu*I (*Pac*I in NEB1 buffer, Biolabs and *Pvu*I in React 7 Buffer, Invitrogen); isolation on agarose gel of the fragment containing the cassette pCMV-NS3-NS4
- Enzymatic digestion of the plasmid pTG6624 (Figure 1C) by *Cla*I (in React 1 Buffer, Invitrogen)
- Bacterial transformation (strain BJ, (Transgene) in order to carry out the homologous recombination between the two plasmid fragments
- Selection of bacterial clones on LB medium + ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis
- Restriction analysis: digestion by *Sma*I and obtaining of fragments of: 2263, 621, 3814, 214, 2164, 909, 180, 2463, 6480, 1398, 4456, 1455, 3540, 3386, 230 and 3685 pb
- Obtaining of the complete adenoviral genome Adenovirus AdNS3NS4, deleted from its E3 and E1 regions, the latter having been replaced by the expression cassette pCMV-NS3-NS4 (pIV317, Figure 1D).

3. Preparation of the adenovirus AdNS3NS4NS5b

This adenovirus allows the expression of the gene coding for the polyprotein NS3/NS4 under the control of the CMV promoter and the expression of the gene coding for the polypeptide NS5b under the control of the SV40 promoter.

3.1 Construction of the transfer plasmid allowing the cloning in the E3 region of the adenovirus of a coding sequence under the control of the CMV promoter

The following stages were implemented:

- Enzymatic digestion of the plasmid pTG4664 (Figure 1E, Transgene) by *Bgl*II (in React 3 Buffer, Invitrogen)
- Enzymatic digestion of the plasmid pTG3074 (Figure 1F, Transgene) by *BamHl*/*Bgl*II (in React 3 Buffer, Invitrogen)
- Ligation (T4 DNA ligase), bacterial transformation (strain 5K)
- Selection of bacterial clones on LB medium + ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis
- Restriction analysis: digestion by *Sma*I and obtaining of fragments of: 4940, 1305 and 230 pb
- Obtaining of the plasmid plV267 (Figure 1G)
- Digestion of the plasmid plV267 thus obtained by *Cla*I/*Mun*I (in React 1 Buffer, Invitrogen)
- Treatment by DNA Polymerase I, Large (Klenow) Fragment (in React 2 Buffer, Invitrogen)
- Ligation (T4 DNA Ligase)
- Bacterial transformation (strain 5K)
- Selection of bacterial clones on LB medium + ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen)
- Restriction analysis: digestion by *Sma*I and obtaining of fragments of: 4692,1305 and 230 pb
- Obtaining of the plasmid plV270, transfer plasmid allowing the cloning in the E3 region of the adenovirus of a coding sequence under the control of the CMV promoter (Figure 1H).

3.2 Replacement of the CMV promoter by the SV40 promoter in plV270

The following stages were carried out:

- PCR amplification of the nucleotide fragment corresponding to the SV40 promoter, from the commercial plasmid pcDNAHygro (Clonetech) using the following oligonucleotides:

- olV232: 5'-GGG GGG AGA TCT CCA GCA GGC AGA AGT ATG-3' (SEQ ID No. 11)

- olV233: 5'-GGG GGG GTC GAC CGA AAA TGG ATA TAC AAG CTC-3' (SEQ ID No. 12)

and according to the procedure described in point 2.1 above, except that a temperature of 58°C instead of 62°C was used

- Enzymatic digestion of plV270 by *Bgl*II/*Sal*I (in React 10 Buffer, Invitrogen)
- Enzymatic digestion of the PCR fragment by *Bgl*II/*Sal*I
- Ligation (T4 DNA ligase), bacterial transformation (strain 5K)
- Selection of the bacterial clones on LB medium + ampicillin (l00 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis
- Restriction analysis: digestion by *Sma*I and obtaining of fragments of: 4692, 719, 80 and 230 pb
- Obtaining of the plasmid plV330, transfer plasmid allowing the cloning in the E3 region of the adenovirus of a coding sequence under the control of the SV40 promoter (Figure 11).

3.3 Insertion of the PCR fragment NS5b into the transfer plasmid plV330

The following stages were carried out:

- PCR amplification of the nucleotide sequence coding for the protein NS5b (SEQ ID No. 3 and 4) using the following nucleotides:

- olV212: 5'-GGG GGG TCT AGA ATG TCA ATG TCC TAC ACA TGG AC-3' (SEQ ID No. 13)

- olV218: 5'-GGG GGG TCT AGA TTA CCG GTT GGG GAG CAG GT-3' (SEQ ID No. 14)

and according to the procedure described in point 2.1 above, except that a temperature of 60°C instead of 62°C was used

- Enzymatic digestion of the plasmid plV330 obtained above by *Xba*I (in React 2 Buffer, Invitrogen)
- Enzymatic digestion of the PCR fragment by *Xba*I

- Ligation (T4 DNA Ligase), bacterial transformation (strain 5K)
- Selection of the bacterial clones on medium LB + ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis
- Restriction analysis: digestion by *Sma*I and obtaining of fragments of: 4692, 1505, 760, 719 and 230 pb
- Obtaining of the plasmid pIV336, transfer plasmid in the E3 deletion containing the sequence NS5b under the control of the SV40 promoter (Figure 1J)

3.4 Homologous recombination with the recombinant adenoviral genome pIV317in order to obtain the adenovirus of the title

The following stages were implemented:

- Digestion of the plasmid pIV317 obtained in point 2.3 above by *Srf*I (in Universal Buffer,Stratagene)
- Digestion of the plasmid pIV336 obtained in point 3.3 by *Nhe*I/*Sac*II (in Buffer T, Amersham Pharmacia Biotech) and isolation on agarose gel of the fragment containing the cassette pSV40- NS5b
- Bacterial transformation (strain BJ) for carrying out the homologous recombination between the two plasmid fragments
- Selection of the bacterial clones on medium LB + ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis
- Restriction analysis: digestion by *Sma*I and obtaining of fragments of: 6480, 4456, 3814, 3540, 3386, 2739, 2463, 2263, 2164,1455, 1398,1105, 909, 760, 719, 621, 230, 214 and 180 pb
- Obtaining of the desired complete adenoviral genome, deleted from the El region, the latter having been replaced by the expression cassette pCMV-NS3-NS4, and deleted from the E3 region, the latter having been replaced by the expression cassette pSV40-NS5B (plasmid pIV342, Figure 1K).

4 Confirmation of the expression of the antigens inserted into the different adenoviruses

The expression of the HCV antigens encoded by the adenoviruses AdNS3NS4, AdNS5b and AdNS3NS4NS5b was verified by Western blot after infection of Huh7 cells.

As expected, all the antigens were expressed.

Example 2: Preparation of a poxvirus allowing the expression of the proteins NS3/NS4 and NS5b according to the invention

1. MVA Poxvirus

The strain Modified Virus Ankara MVATG N33 was supplied by TRANSGENE S. A. (Strasbourg, France).

2. Preparation of the transfer plasmid allowing the expression of the gene NS3/NS4 under the control of the ph5r promoter

2.1 Construction of the pIV250 vector containing the recombination arms BRG2 and BRD2 of the MVA, as well as the selection gene GPT under the control of the promoter ph5r (MVA), followed by a second promoter ph5r in order to allow the expression of the gene of interest

At this point, the insertion of the fragment ph5r-GPT-BRG3-ph5r (originating from the plasmid pTG9997, Transgene) into the plasmid pTG6018 (Transgene) containing the recombination arms BRG2 and BRD2 is desired.

In order to do this, the following stages were carried out:

- Enzymatic digestion by *BamH*I/*Sac*I (in React 2 Buffer, Invitrogen) of the vector pTG6018 (Figure 2A)
- Enzymatic digestion by *BamH*I, then partial digestion by *Sac*I of the plasmid pTG9997 (Figure 2B)
- Purification according to the QIAGEN protocol of the restriction fragment of 1047 pb that contains the sequence coding for ph5r-GPT-BRG3-ph5r
- Ligation (T4 DNA Ligase), bacterial transformation (strain TG1, Statagene)
- Selection of the bacterial clones on ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis (*EcoR*V + *Hind*III (in React 2 Buffer, Invitrogen): fragments of 246, 439, 476, 826 and 2789 pb; *Sac*I: fragments of 915 and 3861 pb)
- Obtaining of the plasmid aimed at (pIV250, Figure 2C).

2.2 PCR amplification of the nucleotide sequence coding for the polyprotein NS3/NS4

The following oligonucleotides were used:

oIV225: 5'-GGG GGG CTG CAG ATG GCG CCT ATC ACG GCC TA-3' (SEQ ID No. 15)

oIV226: 5'-GGG GGG TCT AGA TTA GCA TGG CGT GGA GCA GT-3' (SEQ ID No. 16)

and according to the procedure described in Example 1, point 2.1 above, except that a temperature of 52°C instead of 62°C was used.

2.3 Insertion of the fragment of PCR NS3-NS4 in the plasmid pIV250

In order to do this, the following stages were carried out:

- Enzymatic digestion of the plasmid pIV250 obtained in point 2.1 above by *Pst*I (in React 2 Buffer, Invitrogen)/*Xba*I
- Enzymatic digestion of the PCR fragment NS3/NS4 by *Pst*I/*Xba*I
- Ligation (T4 DNA Ligase), bacterial transformation (strain TG1)
- Selection of the bacterial clones on ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis: (*Hind*III (in React 2 Buffer, Invitrogen): fragments of 4763 and 2789 pb; *Sph*I (in React 6 Buffer, Invitrogen): 1534 and 5991 pb; *Nco*I (in React 3 Buffer, Invitrogen): 2764 and 4761 pb)
- Obtaining of the transfer plasmid containing the sequence coding for the polyprotein NS3/NS4 under the control of the promoter ph5r (pIV327, Figure 2D).

3. Preparation of the plasmid pIV328 allowing the expression of the protein NS5b under the control of the p7.5 promoter

3.1 PCR amplification of the nucleotide sequence coding for the protein NS5b

The following nucleotides were used:

oIV227: 5'-GGG GGG GTC GAC ATG TCA ATG TCC TAC ACA TGG AC-3' (SEQ ID No. 17)

oIV228: 5'-GGG GGG GCA TGC TTA CCG GTT GGG GAG CAG GT-3' (SEQ ID No. 18)

and according to the procedure described in Example 1, point 2.1 above, except that a temperature of 52°C instead of 62°C was used.

3.2 Obtaining of the plasmid

The following stages were carried out:

- Enzymatic digestion of the PCR fragment coding for NS5b by *Sal*I/*Sph*I
- Enzymatic digestion of pTG186 (Figure 2E, Transgene) by *Sal*I/*Sph*I
- Dephosphorylation of the vector pTG186 (ROCHE alkaline phosphatase)

- Ligation (T4 DNA Ligase), bacterial transformation (strain TG1)
- Selection of the bacterial clones on ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis: (*Hind*III: fragments of 1984, 2627 and 4437 pb; *Bgl*II: fragments of 321, 557, 1361, 1451, 2237 and 3121 pb; *Kpn*I (in React 4 Buffer, Invitrogen): fragments of: 2787 and 6261 pb)
- Obtaining of the transfer plasmid containing the sequence coding for the polypeptide NS5b under the control of the p7.5 promoter (pIV328, Figure 2F).

4. Preparation of the transfer plasmids pIV329 and pIV344 allowing the expression of the gene coding for the polyprotein NS3/NS4 under the control of the ph5r promoter and of the gene coding for the polyprotein NS3/NS4 under the control of the p7.5 promoter

In order to do this the following stages were implemented:

- PCR amplification of the nucleotide sequence coding for the protein NS5b from the plasmid pIV328 obtained in point 3.2 above using the following oligonucleotides:

oIV229: 5'-GGG GGG TCT AGA CCG GTA GTT CGC ATA TAC ATA -3' (SEQ ID No. 19)

oIV218: 5'-GGG GGG TCT AGA TTA CCG GTT GGG GAG CAG GT-3' (SEQ ID No. 14)

and according to the procedure described in Example 1, point 2.1 above, except that a temperature of 52°C instead of 62°C was used.

- Enzymatic digestion of the fragment of PCR by *Xba*I
- Enzymatic digestion of the plasmid pIV327 obtained in point 2.3 above by *Xba*I
- Ligation (T4 DNA Ligase), bacterial transformation (strain TG1)
- Selection of the bacterial clones on ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of 2 positive clones after restriction analysis: (*Pst*I: pIV329: fragments of 3033 and 6466 pb, pIV344: 4641 and 4858 pb; *Apa*I (in React 4 Buffer, Invitrogen): pIV329: 454, 960 and 8085 pb, pIV344: 454, 1418 and 7627 pb; *Nco*I: pIV329: 4269, 469 and 4761 pb, pIV344: 3053, 1685 and 4761 pb; *Sma*I: pIV329: 214, 2164, 1444 and 5677 pb, pIV344: 214, 2164, 928 and 6193 pb)

- Obtaining either of the transfer plasmid allowing the expression of the polyprotein NS3/NS4 under the control of the ph5r promoter and of the protein NS5b under the control of the p7.5 promoter, the 2 expression cassettes being oriented in the same direction (pIV329, Figure 2G), or of the transfer plasmid allowing the expression of the polyprotein NS3/NS4 under the control of the ph5r promoter and of the protein NS5b under the control of the p7.5 promoter, the 2 expression cassettes being oriented in opposite directions (pIV344, Figure 2H).

5. Confirmation of the expression of the antigens inserted into the different poxviruses

It was verified by Western blot, after infection of Huh7 cells with the poxviruses concerned, that the poxviruses pIV329 and pIV344, containing the sequences coding for the polyprotein NS3/NS4 and the polypeptide NS5b, expressed said HCV antigens.

Example 3: Demonstration of the immunogenicity of the combination of NS3/NS4 and NS5b

1. Immunization of mice

HLA-A2.1 transgenic mice were immunized, once, by intramuscular injection of at least one adenovirus chosen from the following adenoviruses:

- AdNS3NS4 prepared in Example 1 above (point 2.3),
- AdNS5 prepared in Example 1 above (point 3.3),
- AdNS5a prepare according to the procedure of Example 1, point 2, except that the following nucleotide primers were used in order to amplify the nucleotide sequence coding for the polypeptide NS5a (SEQ ID No. 5 and 6):

olV172: 5'-GGG GGG GGT ACC ATG TCC GGC TCG TGG CTA AGG-3' (SEQ ID No. 20),

olV173: 5'-GGG GGG TCT AGA TTA GCA GCA GAC GAT GTC GTC-3' (SEQ ID No. 21),

in the PCR the temperature of 62°C was replaced by 56°C, the enzymatic digestion of pTG 13387 and of the fragment NS5a were implemented by *KpnI*/*XbaI*, restriction analysis by digestion by *SmaI* of pTG13387 producing fragments of 180 and 7251 pb and of pTG6624 producing fragments of 2263, 621, 5615, 180, 2463, 6480, 1398, 4456, 1455, 3540, 3386, 230 and 3685 pb.

- AdCE1E2 according to the procedure of Example 1, point 2, except that the following nucleotide primers were used in order to amplify the nucleotide sequence coding for the core-E1-E2 polyprotein (also called CE1CE2) (SEQ ID No. 7 and 8):

olV62: 5'-GGG GGG GCT AGC ATG AGC ACA AAT CCT AAA CCT-3' (SEQ ID No. 22),

olV68: 5'-GGG GGG TCT AGA TCA GGC CTC AGC CTG GGC TAT-3' (SEQ ID No. 23),

in the PCR the temperature of 62°C was replaced by 56°C, the enzymatic digestion of pTG13387 and of the fragment CE1CE2 were implemented by *Nhel/Xba*I, restriction analysis by digestion by *Sma*I of pTG13387 producing fragments of 163, 435, 2270, 180 and 5254 pb and of pTG6624 producing fragments of 2263, 621, 3618, 163, 435, 2270, 180, 2463, 6480, 1398, 4456,1455, 3540, 3386, 230 and 3685 pb,

- AdNS3NS4NS5b prepared in Example 1 above (point 3) and
- AdβGal (Transgene),

according to the following protocol:

- 10^9 pfu of AdNS3NS4 or
- 10^9 pfu of AdNS5b or
- 10^9 pfu of AdCEIE2 or
- 10^9 pfu of AdNS3NS4 and 10^9 pfu of AdNS5b or
- 10^9 pfu of AdNS3NS4, 10^9 pfu of AdNS5b and 10^9 pfu of AdNS5a
- 10^9 pfu of AdNS3NS4, 109 pfu of AdNS5b and 10^9 pfu of AdCEIE2
- 10^9 pfu of AdNS3NS4 NS5b or
- 10^9 pfu of Adβ-Gal as control.

Before immunization, the expression of the HCV and β-Gal antigens by the different adenoviruses used for the immunization were verified by Western blot.

2. CTL and ELISPOT Tests

Fifteen days after injection, the cell response was analyzed by isolating the spleen cells (splenocytes) of the mice and a CTL test and an ELISPOT test were carried out as follows:

For the CTL test, these splenocytes were cultured on 24-well plates in the presence of:

- 5 µM of the epitope GLL (GLLGCIITSL, SEQ ID No. 24) in the case of the splenocytes originating from mice having received AdNS3NS4, 5 µM of the epitope ALY (ALYDVVSTL, SEQ ID No. 25) or 5 µM of the epitope KLQ (KLQDCTMLV, SEQ ID No. 26) in the case of the splenocytes originating from mice having received AdNS5b or 5 µM of the epitope DLM (DLMGYIPLV, SEQ ID No. 27) in the case of the splenocytes originating from mice having received AdCE1E2, said epitopes being in synthetic peptide form (Eurogentex) and,

- 10 U of murine recombinant interleukin 2 (Brinster et al., Hepatology 2001) per ml in alpha minimum essential medium (αMEM) for 5 days. On the 5th day, the restimulation stage was carried out, which consists of adding naive mice splenocytes to the splenocytes in culture in the presence of said epitopes over 2 days. On the 7th day, the CTL test was carried out, which consists of bringing into contact the splenocytes from the immunized mice after 7 days of culture (effector cells) and EL4 S3-Rob HDD cells loaded with 10 µM of said epitopes and labelled with Cr^{51} (target cells). The specific cytotoxic activity of the effector cells was determined by measuring, after 4 hours of incubation with the target cells, Cr^{51} released following lysis of the target cells using a γ-Cobra II counting apparatus (Packard, Rungis, France) The maximum spontaneous release from wells containing either medium alone, or lysis buffer (HCl IN) was determined. The specific percentage of cytotoxicity was calculated by the formula:

(release in the test - spontaneous release)/(maximum release - spontaneous release) x100. The epitope-specific lysis was determined by the difference between the percentage of specific lysis obtained in the presence or in the absence of said epitopes.

The ELISPOT test was carried out by culturing the splenocytes for 48 hours in Multiscreen 96-well plates (Millipore) previously coated with anti-interferon gamma antibodies (IFNγ) (l0 µg/ml final). The splenocytes were cultured in the presence of 10 µM of the appropriate epitopes, as indicated above, and of 10 U of murine recombinant interleukin 2 per ml in αMEM. For the positive control, the splenocytes were cultured in the presence of concanavalin A (5 µg/ml). For the negative control, the splenocytes were cultured either in the presence of a non-specific peptide belonging to the

capsid protein of HCV, of sequence DLMGYIPLV (also called irrelevant peptide), or in medium alone without epitope. The wells were washed three times, with 0.05% PBS-Tween then PBS respectively, an operation followed by incubation for 2 hours with anti-IFNγ antibodies from biotinylated mice. After washing, the wells were incubated for 1 hour with a streptavidine-horseradish peroxidase conjugate and the enzymatic activity was developed by degradation of the AEC (aminoethylcarbazole) substrate. The spots obtained were counted using a Zeiss ELISpot reader (Zeiss microscope in conjunction with the KS-ELISpot software).

The results are indicated in Figures 3 to 5 in which M corresponds to mouse and Neg. mouse corresponds to the control mouse.

These results demonstrate that

- AdNS3NS4 clearly induces a cell-mediated response specific of the expressed antigens, as illustrated in Figure 3A and 3B by the detection of T lymphocytes specific to the epitope GLL contained in NS3.
- AdNS5b clearly induces a cell-mediated response specific of the expressed antigens, as illustrated in Figure 4 by the detection of T lymphocytes specific to the epitope ALY and KLQ contained in NS5b.
- AdCEIE2 clearly induces a cell-mediated response specific of the expressed antigens, as illustrated in Figure 5 by the detection of T lymphocytes specific to the epitope DLM contained in the Core protein.

3. *In vivo* trial test using a recombinant vaccinia virus

In order to evaluate whether the specific immune responses induced by the different adenoviruses were capable of inducing protection against a infectious disease trial ("*in vivo* protection"), we subjected the vaccinated mice to such a trial.

The mice not being directly infectable by HCV, in order to link the induction of a specific immune response and resistance to an infection, we used a recombinant vaccinia virus (strain WR) coding for the non-structural proteins of HCV (NS2 to NS5b) in order to carry out this trial. This recombinant vaccinia virus, after intra-peritoneal injection of 10^7 pfu in the mouse, will be replicated in the animal. The replication of this virus induces an immune response both specific to the vaccinia antigens and specific to the HCV antigens, as it also expresses the NS proteins of HCV. This specific

response to the HCV antigens will be all the more effective and vigorous as the mice will have already received a vaccine expressing the HCV antigens. In other words, the more the effective vaccination (in the present case carried out with the recombinant adenoviruses) has been (i.e. the immune system of the mice have been effectively "primed" by the vaccine), the stronger will be the anti-HCV response generated after trial by the recombinant vaccinia virus and, consequently, the more the mice are "protected" against this trial. In practice, the lower the residual vaccinia virus count in the mice, the more effective the protection or the neutralization due to the vaccination has been.

The neutralization of the vaccinia virus reflects both the cell response induced by the HCV proteins and by the vaccinia proteins. The neutralization is evaluated by titration of the residual vaccinia virus from the ovaries of the animals as follows: the ovaries are removed 4 days post-trial, sonicated, freeze-thawed 3 times then after centrifugation, successive dilutions of supernatant are titrated according to the lysis plaque technique (Murata et al., PNAS, vol. 100, p. 6753-6758) on Hutk- cells. The viral titres are determined in pfu/ml/mg of ovary.

4. Demonstration of superior protection of a vaccination combining the polyprotein NS3/NS4 and the polypeptide NS5b.

The recombinant virus titre of the vaccine was determined for 4 groups of 8 mice immunized by the following combinations of adenoviruses: AdNS3NS4 + AdNS5b (1st group), AdNS3NS4 + AdNS5b + AdNS5a (2nd group), AdNS3NS4 + AdNS5b + AdCEIE2 (3rd group) and AdβGal (4th group).

The results, given in Figure 6, are treated statistically on the basis of the Wilcoxon Mann-Whitney non-parametric test (Méthodes Statistiques à l'usage des médecins et des biologistes, Collection Statistique en Biologie et en Médecine, Flammarion Medecine Sciences, (D. Schwarz), 1977), which is based on a comparison of the averages, and allows the comparison of the values of two independent samples *x* and *y*.

This test is implemented as follows: all of the values of the two groups *x* and *y* to be compared are classified in increasing fashion. A rank is then allocated to each value, and the sum of the ranks is calculated. W*x* and W*y* are then obtained. A reference value called $(Wx)_t$ (theoretical value in the null hypothesis where W*x* is not different from W*y*) is then calculated and linked by

the ratio: n (N+1)/2, with n = number of mice tested in group $_x$ and N = number of mice tested in groups *x* and *y*.

If W_x is less than $(Wx)_t$ (low residual level of vaccinia virus in the mice), then it can be concluded that the neutralization resulting from the vaccination is significantly effective.

If we take the example of the group AdNS3NS4S5b denoted *x* compared with the group AdβGal denoted *y*, we obtain the following values:

$Wx = 1+2+4+6+8+11+13+14 = 59$ (8 mice tested)

$Wy = 3+5+7+9+10+12+15+16 = 77$ (8 mice tested)

Under the null hypothesis, W*x* is not different from W*y*, the expected value is: $(Wx)_t = (1/2) *8*17 = 68$

$Wx < (Wx)_t$, which signifies that the values obtained in the group AdNS3NS4NS5b are smaller than those obtained in the group AdβGal and that the neutralization resulting from the vaccination is significantly effective.

The statistical values for the other groups of mice are indicated in Table 1 below:

Table 1

Group/AdβGal	Wx	$(Wx)_t$
AdNS3NS4+ NS5b	52	68
AdNS3NS4+ NS5b+ NS5a	68	68
AdNS3NS4+ NS5b+ CE1E2	74	68

The values in Table 1 above show that only a vaccination of the mice by a combination of the Adenoviruses NS3NS4 and adenovirus NS5b is capable of inducing a significant neutralization of the replication of the vaccinia virus used in the trial with respect to the group of control mice vaccinated by AdβGal. The vaccinations carried out using the combinations comprising (AdNS3NS4 + AdNS5b + AdNS5a) or (AdNS3NS4 + AdNS5b + AdCE1E2), do not result in a significant difference compared with the group of control mice immunized by AdβGal.

These results therefore make it possible to demonstrate, unexpectedly, the superior protection of a vaccination combining the polyprotein NS3NS4 and the polypeptide NS5b.

5. Confirmation of the protection of a vaccination combining the polyprotein NS3NS4 and the polypeptide NS5b expressed jointly by the same vector

The recombinant vaccinia virus titre was determined for 3 groups of 8 mice immunized by the following combinations of adenoviruses: AdNS3NS4AdNS5b (1st group), AdNS3NS4 + AdNS5b (2nd group), and AdβGal (3rd group).

The results, given in Figure 7, are treated statistically on the basis of the Wilcoxon Mann-Whitney non-parametric test as described in the previous experiment.

The statistical values for groups 1 and 2 compared to the control group AdβGal are indicated in Table 2 below:

Table 2

Group/AdβGal	Wx	$(Wx)_t$
AdNS3NS4NS5b	49	68
AdNS3NS4+ NS5b	53	68

The values in Table 2 above show that the vaccination of the mice by an adenovirus coding both for the three antigens NS3, NS4 et NS5b, like the combination of the Adenovirus NS3NS4 and Adenovirus NS5b, is capable of inducing a significant neutralization of the replication of the vaccinia virus used in the trial with respect to the group of control mice vaccinated by the AdenoβGal. This result confirms the protection of a vaccination combining the polyprotein NS3/NS4 and the polypeptide NS5b expressed jointly by the same vector.